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Nuclei are given support and structure by a network of proteins and filaments called the nuclear lamina. Mutations in many genes encoding lamina components result in human diseases known as the laminopathies. Hutchinson-Gilford Progeria Syndrome (HGPS) occurs from a rare mutation in a major lamina component. Patients exhibit aspects of rapid aging including artherosclerosis, osteoporosis and sclerderma. HGPS is also associated with a nuclear dysmorphology in which multiple protrusions alter the normal shape of the nucleus, possibly causing the rapid aging phenotypes.

A mutation of the *D. melanogaster* gene *dtopors* phenocopies this nuclear dysmorphology. The dTopors protein is a component of the lamina in all cell types examined, but unlike in HPGS, visible nuclear defects are limited to male germline cells.

Here, I investigated both the germ line and soma of *dtopors* male flies to determine if rapid aging occurs. Results indicate that *dtopors* males lose the ability to produce progeny at a younger age than wildtype. Testes size decreases at a younger age, but is not due to decreased stem cell numbers. Somatic cells also appeared to be affected, as lifespan was shortened, and an enhanced age-related decrease in negative geotaxis was observed in *dtopors* males. Tests of effects on an age-related decrease in innate immunity yielded ambiguous results. Taken together, the results suggest that acceleration of some aspects of aging may be induced by mutations in *dtopors*, and that study of dTopors in *Drosophila* could yield insight into similar accelerated aging processes that coincide with changes in nuclear structure.

EFFECTS OF *DTOPORS* ON SOMA AND GERM LINE AGING IN MALE *DROSOPHILA*

by

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Approved by

_____
Committee Chair

To my wife Philna, I had no idea that elevator ride would take me this high.

If I had a chance for another try,
I wouldn't change a thing,
It's made me all of who I am inside,
And when I do thank God
that I am here, and that I am alive,
And every day I wake,
I tell myself a little harmless lie,
The whole wide world is mine.

- Tom Delonge – Angels and Airwaves

APPROVAL PAGE

This thesis written by Christopher Hylton has been approved by the following committee of the Faculty of The Graduate School at the University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

The nuclear lamina is composed of a network of various proteins and filaments associated with the inner face of the nuclear envelope that both provide mechanical support and enable communication between the nucleus and cytoplasm. Mutations in many genes encoding components of the nuclear lamina result in human diseases collectively known as the laminopathies. These can include nonsense and missense mutations, gene dosage effects (Eriksson, Brown, et al. 2003), processing defects, and splicing defects. Development of autoantibodies, which may also interfere with the functions of lamina proteins, can also result in laminopathies (Padiath, Saigoh, et al. 2006). Perhaps the most striking of these laminopathies is known as Hutchinson-Gilford Progeria Syndrome (HGPS), in which rare mutations occur in a major lamina component, lamin A (Eriksson, Brown, et al. 2003).

HGPS, characterized by premature aging (Baker, Baba, et al. 1981), causes death in at least 90% of patients by the age of 13. The cause of this phenotype is a *de novo* single base substitution in *LMNA* gene that codes for both Lamin A and Lamin C (Eriksson, Brown, et al. 2003). The lamins assemble into intermediate filaments responsible for the construction and stability of the nuclear lamina. This laminar stability is essential for chromatin attachment, DNA replication, and overall nuclear organization (Eriksson, Brown, et al. 2003). Children often show no phenotypic clues of HGPS at

birth, but early in life, patients begin to express phenotypes typically seen in old age, such as growth retardation, alopecia, sclerodermatous skin, hypoplasia of bones and osteoporosis (Varga, Eriksson, et al. 2006). Progressive signs of premature aging continue into the early teens where death usually occurs by heart attack or stroke as a result of artherosclerosis of the coronary arteries (Baker, Baba, et al. 1981).

Mouse knock-out studies show that a deletion of the prelamin processing enzyme *Zmpste24* also causes premature aging (Mounkes, Kozlov, et al. 2003) along with higher rates of aneuploidy, balloon shaped chromatin and chromosomal instability in bone marrow cells as early as four weeks of age. Additionally in mouse, unprocessed lamin A caused by mutations in *Zmpste24* results in more DNA damage, higher sensitivity to DNA damage, and accumulation of unprocessed lamin A. These results suggest that improper lamina construction impedes the recruitment of proteins required for DNA response and repair, which closely parallels mechanisms in progeria-like syndromes that arise from mutations in DNA repair enzymes (Liu, Wang, et al. 2005).

In progeria patients, the lamin A precursor, prelamin A, lacks a cleavage site that removes the last 15 AA of the prelamin allowing for insertion into the nuclear lamina (Hennekes and Nigg, 1994). This lack of processing leaves the protein, now referred to as progerin, anchored in the nuclear membrane causing the cascade of events leading to premature aging (Capell and Collins, 2006). The structural hypothesis implies that *Lmna* mutations cause the nucleus to be more fragile resulting in cell death and progressive disease in mechanically stressed tissues (Zwerger, Ho, et al. 2011), yet HGPS cells exhibit stiffer nuclei as a possible result of progerin buildup at the nucleus (Dahl,

Scaffidi, et al 2006). The gene regulation hypothesis instead states that a disrupted interaction with tissue-specific transcription factors causes the onset of different disease phenotypes (Worman, Fong et al. 2009). Epidermal stem cells depletion is seen in mice with HGPS (Rosengradten, McKenna et al. 2011), and it is proposed that increased turnover and abnormal differentiation of adult stem cells along with increased mechanical sensitivity may be the cause of stem cell death in laminopathies. Identification of additional models may assist in answering these enigmas of the nuclear lamina.

At the cellular level, HGPS causes a nuclear dysmorphology in which multiple protrusions, or blebs, alter the normal shape of the nucleus. It remains unknown if a direct relationship between perturbations in nuclear shape and rapid aging phenotypes exists. Alterations in nuclear shape and premature aging have been described in mutations of genes that do not encode lamina components such as the SWI/SNF chromatin remodeling enzyme ATPase BRG1, which is suggested to control nuclear shape by internal nuclear mechanisms that control chromatin dynamics (Imbalzano, Cohet et al. 2013), and the DNA repair nuclease ERCC1-XPF, which causes a progeria like phenotype but is poorly understood (Choi, Wang et al. 2011). Since mutations in BRG1 and ERCC1-XPF have both been associated with rapid aging phenotypes, this may indicate that there is a unique relationship between lamina structure and aging, perhaps related to chromatin organization at the lamina.

***dtopors*: A Drosophila model for Progeroid disease?**

Here, I am interested in examining mutations in a gene in the male fruit fly *Drosophila melanogaster* that in some ways phenocopy aspects of HPGS. This gene, called *dtopors* (Drosophila Topoisomerase I-binding arginine/serine-rich protein) results in nuclear dysmorphology similar to that of HGPS. The dTopors protein is a component of the nuclear lamina (Capelson and Corces, 2005), and is expressed in all cell types examined, yet nuclear shape changes in *dtopors* mutants seem to be limited to male germline cells (Matsui, Sharma et al. 2011). This unique role for dTopors in spermatocytes remains unclear.

The t*opors* gene is conserved in multicellular eukaryotes, and homologs have been identified in mammal including human and mouse. Human Topors (hTopors) was first isolated and identified by an *in vitro* screen for topoisomerase I-binding proteins and alteration of function in humans (Haluska, Saleem et al. 1999). hTopors was also identified as p53BP3, a protein that interacts with p53 (an important component in cell-cycle control, gene regulation, and tumor suppression) *in vivo* (Zhou, Wen et al.1999), and LUN, a protein encoded by an mRNA highly expressed in normal human lung tissue (Oyanagi, Takenaka et al. 2004). Fly, mouse, and human Topors all contain a single RING finger domain at the amino-terminus, two bipartite nuclear localization sequences (NLS) near the middle of the protein (Haluska, Saleem et al. 1999), numerous PEST sequences present at the N terminus, C terminus, and middle of the protein, an arginine/serine rich (RS) region of unknown function (Zhou, Wen et al. 1999; Chu, Kakazu et al. 2001; Secombe and Parkhurst 2004) (Figure 1). There is high conservation

of the RING domain of both dTopors and hTopors with members of the viral ICP0 protein family (Weger, Hammer et al. 2002). The RING finger domain is associated with DNA-binding and ubiquitin ligase activity (Rajendra, Malegaonkar et al. 2004). The PEST domains are involved in protein degradation, suggesting that Topors has a short half-life (Zhou, Wen et al. 1999).

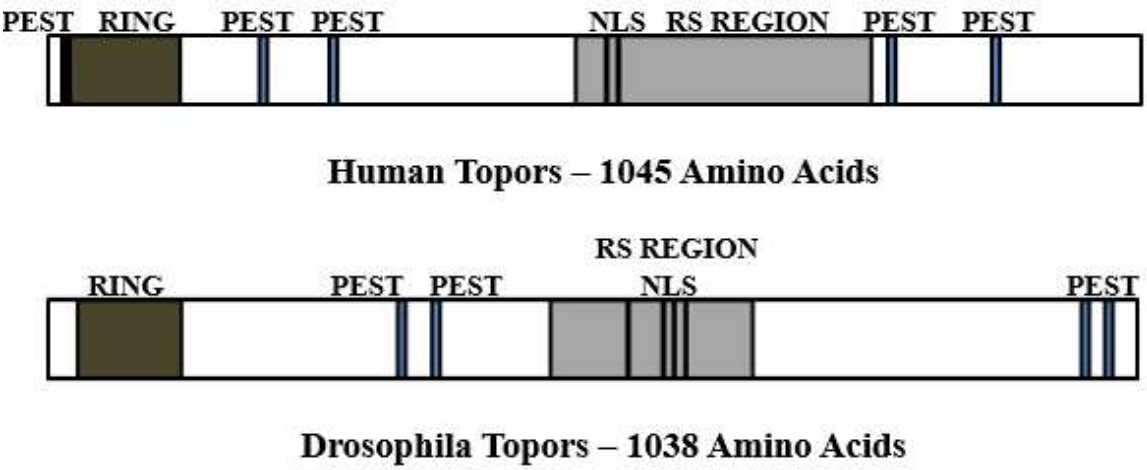

Figure 1. hTopors and dTopors domain structure comparison. (Modified from Secombe and Parkhurst 2004)

hTopors encodes 1045 amino acids (Weger, Hammer et al. 2003) and maps to the short arm of chromosome 12 (Zhou, Wen et al. 1999). hTopors is ubiquitously expressed in humans somatic and germline tissues (Chu, Kakazu et al. 2001). In addition to interaction with transcription-regulating Topoisomerase I, a yeast two-hybrid screen showed hTopors interacts with two adeno-associated virus AAV-2-REP proteins enhancing AAV-2 gene expression (Weger, Hammer et al. 2002) and with p53 (Zhou, Wen et al. 1999). Both *in vitro* and murine *in vivo* studies showed that hTopors functions as an E3 ubiquitin ligase in a RING domain-dependent manner and both mono- and

polyubiquitinates p53, leading to its proteasome-dependent degradation (Rajendra, Malegaonkar et al. 2004). hTopors immunoprecipitates with p53 in COS -7 cells and overexpression of mTopors enhances the p53-dependent growth suppression of tumor cells in H1299 cell line by stabilizing p53 and increasing its ability to limit cell growth (Lin, Ozaki et al. 2005). Contradictory to hTopors ubiquitinating p53 for destruction, overexpression of hTopors stabilizes p53 possibly by resulting in the interference of the ubiquitination pathway or activation of sumoylation pathway.

hTopors was the first protein identified to have dual SUMO-1 and ubiquitin E3 ligase activity (Weger, Hammer et al. 2003; Rajendra, Malegaonkar et al. 2004). A RING domain-independent hTopors can both sumoylate a target protein and be sumoylated itself on one of three specific lysine residues (Weger, Hammer et al. 2003). Known targets of Topors sumoylation include multiple sites on topoisomerase I (Hammer, Heilbronn et al.2007), chromatin modifying and transcription regulating proteins (Pungaliya, Kulkarni et al. 2007), and IKKe which is activated in response to DNA damage (Renner, Moreno et al. 2010). Topors ubiquitinates the transcription factor NKX3.1 which slows the cell cycle and cell growth in prostate tissue (Guan, Pungaliya et al. 2008), and it also ubiquitinates H2AX which leads to phosphorylation of H2AX, an epigenetic marker for DNA damage (Seon, Nam et al. 2012). Phosphorylation of serine 98 on hTopors abolishes the ubiquitination activity of hTopors while rendering no effect on sumoylation activity suggesting the phosphorylated serine functions as a switch for hTopors effect on substrates (Park, Zheng et al. 2008). Furthermore, the

phosphorylation of serine residues on hTopors is regulated by Polo-like kinase 1 (PLK-1), (Yang, Li et al. 2009) leading to its destruction (Yang, Li et al. 2012).

In flies, dTopors functions as an E3 ligase both *in vitro* and *in vivo* but activity in sumoylation remains has not been demonstrated. One of its ubiquitination targets is Hairy, a transcriptional repressor involved in embryonic pattern formation. dTopors directs ligation of ubiquitin chains on Hairy, marking it for destruction by the proteasome (Secombe and Parkhurst 2004). An additional function unique to dTopors is the ability of the RING domain to interact with two of the three major protein component of *gypsy* chromatin insulators, improving their enhancer blocking and gene shielding activities (Capelson and Corces 2005). dTopors has been shown to regulate *gypsy* insulators in a dose-dependent manner. Insulator function is perturbed when dosage of dTopors is reduced (Capelson and Corces 2005; Matsui, Sharma et al. 2011), but surprisingly flies in which dTopors is eliminated show wildtype insulator activity in genetic assays (Matsui, Sharma et al. 2011). Knockouts of *gypsy* insulator components known to bind chromatin are rescued by *dtopors* overexpression, suggesting that dTopors may play a role in the binding of *gypsy* insulator complexes to their target sequences (Capelson and Corces 2006).

In addition to possible differences in function, the fly and mammalian Topors also show some differences in intracellular localizations. Staining of hTopors in lung carcinoma cells reveals a punctate nuclear localization that correlates to PML (Promyelocytic leukemia) nuclear bodies (NBs). PML NBs are structures in the nucleus that include many proteins involved in growth, apoptosis, and transcription-regulating

proteins, and the PML protein is required for hTopors to bind to the PML body (Rasheed, Saleem et al. 2002). hTopors induces the relocalization of SUMO-1, SUMO-1 bound target proteins (Weger, Hammer, et al. 2005), NKX3.1 (Guan, Pungaliya et al. 2008), and IKK-e (Renner, Moreno et al. 2010) to these NB protein storehouses in the cell. The exact function of PML bodies remains unknown. hTopors dissociates from NBs to a diffuse pattern in the nucleus with the addition of the transcriptional inhibitor DRB and the apoptosis-inducing compound camptothecin (Rasheed, Saleem et al. 2002) suggesting hTopors may have an additional role in DNA damage repair pathways.

hTopors mRNA and protein expression is readily detected in most human tissues with highest quantities in the colon, lung, kidney and uterine tissue. Colon adenocarcinoma samples express lower levels of *hTopors* mRNA and hTopors while regular and benign adenoma colon tissue express normal levels of hTopors (Saleem, Dutta et al. 2004), and a similar result is seen in mouse when mutated murine *topors* (*mtopors*) caused a 7-fold increase in tumor development (Marshall, Bhaumik et al. 2010). *mtopors* mutant embryonic fibroblasts, pMEFs, display aneuploidy possibly linked to mislocalization of HP1, which is required for cohesin recruitment to centromeres. *mtopors* male and female mice are fertile suggesting mTopors is not needed for germ line production, yet *mtopors* mice have increased perinatal mortality, decreased weight, and decreased lifespan (Marshall, Bhaumik et al. 2010).

A recent study using normal murine mammary gland (NMuMG) cells and embryonic murine dorsal epidermis cells found mTopors to not only localize to punctate spots in the nucleus, but also to the cellular periphery in the cytoplasm of mitotic cells.

mTopors localizes with Sdc-1, a regulator of cell growth and regulator of proteins involved with retinal deficiencies (Braun and DeWispelaere et al. 2012).

The localization of mammalian Topors also shows tissue and cell type specificity. Specific *htopors* mutations have been linked to autosomal dominant retinitis pigmentosis (adRP), resulting in night blindness, peripheral vision loss, and eventual total vision loss, a disorder affecting approximately 1.8 million worldwide (Chakarova, Papaioannou et al. 2007). There have been five *htopors* mutations identified that result in this phenotype, and all mutations result in premature stop codons and code for incomplete, nonfunctional hTopors (Bowne, Sullivan et al. 2008). In pig, mouse and human retina photoreceptor cells, Topors localizes to the connecting cilium junction of the photoreceptor inner and outer segments indicating a role in periciliary machinery needed for cargo transport, yet in retinal ganglion cells, Topors localizes to punctate spots in the nucleus except during mitosis, where they associate with the centrosome (Chakarova, Khanna et al. 2011).

In Drosophila, there are two main differences in localization compared to its mammalian counterparts. First, dTopors colocalizes with the nuclear lamins Dm0 and C in both somatic and germline cells (Capelson and Corces 2005; Matsui, Sharma et al. 2011), and second, dTopors localizes to gypsy insulator bodies, which are chromatin bound structures. The one similarity in localization is that dTopors is also seen in punctate nuclear spots reminiscent of the localization to PML bodies in mammals, but this localization is limited to late prophase spermatocytes in the male germ line (Matsui, Sharma et al. 2011).

dtopors Phenotypes in Spermatogenesis and Aging

The male germ line in *dtopors* mutant flies expresses a tissue-specific phenotype resulting in improper lamina assembly and meiosis I chromosome transmission errors. The number of progeny per *dtopors* male is significantly less than produced by wildtype males (Matsui, Sharma et al. 2011), and *dtopors* spermatocytes exhibit nuclear blebbing likely a result of a disrupted nuclear lamina.

Several lines of evidence suggest that dTopors function in male meiosis may be dependent on its association with the lamina. Examination of testis squashes from *dtopors* males revealed that spermatocytes have abnormal finger-like protrusions (blebbing) of the nuclear lamina and an uneven perinuclear space (Matsui, Sharma et al. 2011). Antibody staining to both *Drosophila* lamin types, lamin Dm0 and lamin C, show that in *dtopors* males both lamins still localize to the nuclear lamina; however, lamin staining of both types is abnormally distributed throughout the intranuclear foci (Matsui, Sharma et al. 2011). Quantitative western analysis indicates that the nuclear blebbing is not caused by changes in lamin protein abundance, but rather in the complete localization and assembly of lamin into the nuclear lamina at the nuclear membrane. Additionally, *dtopors* males exhibit a variety of meiotic defects including chromatin condensation defects, anaphase bridges, centriole separation defects, and aneuploidy. These phenotypes might be attributable to an unresolved chromatin/dTopors/lamina interaction leading to a meiotic temporal defect or influencing stem cell fate.

Cursory examination of *dtopors* male flies suggested that they may exhibit a premature aging phenotype in the germ line (M. Matsui and J. Tomkiel, personal

communication). Here, I more thoroughly investigate *dtopors* flies to see if aging is altered in a similar manner to the human progeria disease. Drosophila is an opportune model organism in that numerous assays and reagents exist to assay both somatic and germline aging. These tools may be beneficial to determine the relationship between the nuclear dysmorphology and aging. I want to see if the premature aging is limited to the germ line where the nuclear shape changes are observed, with particular interest in stem cells.

CHAPTER II

AGING IN *DTOPORS* MALE FLIES

Materials and Methods

Drosophila culture and stocks

The Z1837 and Z4522 *dtopors* alleles originated from a collection of viable, second chromosome ethyl methane sulfonate-induced mutations (Koundakjian et al. 2004) and were identified by virtue of high frequencies of chromosome 4 loss among progeny of mutant fathers (Wakimoto et al, 2004). The *dtopors* deletion allele, *Df(dtopors^{AA})* is courtesy of Susan Parkhurst. All other stocks were acquired from the Bloomington Stock Center (www.flybase.org). All crosses and stocks were maintained on standard fly food composed of cornmeal, molasses, yeast, agar medium at 25°C.

Dissection and tissue preservation

Flies were dissected in Schneider's Insect Medium (Fisher, Waltham, MA). Testes were fixed in 4% formaldehyde in Phosphate Buffered Saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) for phase microscope analysis or prepared for indirect immunofluorescence as described below.

Testes phase contrast images

Formaldehyde-fixed testes were mounted on slides prepared with two layers of Scotch Magic tape on either side of the top of the slide in order to not squash the tissue with the coverslip. Testes from males of various ages of five genotypes were analyzed. Images of individual testes were captured on a Nikon Eclipse E600 phase microscope with a RT color camera (Diagnostic Instruments, Model # 2.2.1).

Indirect immunofluorescence and confocal microscopy

Testes were collected in Schneider's media and rinsed one time in PBS, then fixed in ice-cold methanol for ten min and rinsed three times with 1X PBS. Primary antibodies (Iowa Hybridoma Bank, Iowa City, Iowa) were diluted with 1X PBS with 0.1% Triton X (PBST) + 1% bovine serum albumin (BSA) to the following concentrations: rat anti-vasa 1:200 (anti-vasa), mouse anti-fasciclin III 1:50 (7G10). Testes were incubated in primary antibodies overnight at 4°C. The following day, the testes were washed in PBST three separate times for thirty min each. Alexa Fluor 546 goat anti-rat and Alexa Fluor 488 goat anti-mouse secondary antibodies (Life Technologies, Carlsbad, CA) were diluted 1:1000 in PBST + BSA. Testes were incubated in secondary antibodies overnight at 4°C and washes repeated. The cells were stained with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI, Sigma) for one minute and mounted in 50% glycerol in PBS. For each primary antibody, a minimum of five pairs of testes of each genotype were examined per age group.

Longevity assay

Flies were collected less than 24 hrs after eclosion. Vials of ten male flies each were set up and a total of 500 male flies for each genotype were monitored. Every five days, the flies were carefully transferred to a new vial of fresh food without using CO_2. Survivors were scored every fifth day until forty days of age.

Fertility assay

Ten male flies of each genotype were collected less than 24 hrs after eclosion. Wildtype female flies were removed less than eight hrs after eclosion to ensure virginity. Ten vials per genotype were set up with an individual male and five wildtype virgin females less than two days old. Every three days, the male fly was removed from the vial under CO_2 anesthesia and placed in to a new vial with fresh food and five new virgin females less than two days old. The females were allowed to lay eggs until Day 11 then removed. Progeny of the cross were scored until Day 18. Males are transferred every three days.

Negative geotaxis assay

A modification of a behavioral assay by D. Leffelaar and T. Grigliatti. (1984) was used to assay the flies innate escape response to run to the top of a tube and sit upside down. A single male fly was placed in a 15 cm test tube with a line, 10 cm from the bottom of the tube, marked around the perimeter. The tube was tapped to knock the fly down to the bottom of the tube and a timer was initiated. If the fly did not cross the 10

cm mark after two min, the trial was recorded as "did not complete." Fifty trials of each genotype at each age were recorded.

Innate immunity assay

Five ml cultures of bacteria were grown overnight, centrifuged and resuspended in 50 µl LB. Ampicillin resistant *E.* coli were grown in LB plus 100 µg/ml ampicillin. *S. aureus* were grown in LB. Using a 31 gage needle, flies were inoculated via microinjection into the center of the prescutum on the dorsal side of the thorax. Flies that died within 1 hr of injection were not scored. Flies were scored for survival after 24, 48, and 72 hrs. Control flies of each genotype were injected with an empty needle.

Statistics

Survival data were analyzed using 2 x 2 contingency tests to determine if differences between were significant at each time point. For tests of fertility, groups were compared using Student's T test.

Results

Testis size in dtopors males decreases at an earlier age

Cursory observations from M. Matsui and J. Tomkiel suggested that testes size in *dtopors* males seemed to shrink in size at a much younger age than that of wildtype male flies. Using phase contrast microscopy, I saw that testes size seems to be diminished in

dtopors males at an age as young as seven or eight days old. This reduction is not seen in male wildtype flies until somewhere between 30 and 40 days of age.

I analyzed at least ten male flies of the following genotypes: wild type (Canton S) +/+; homozygous for the a piggybac transposon $dtopors^{f05115}$ /$dtopors^{f05115}$; homozygous for a deletion of dtopors, $Df(dtopors^{AA})/Df(dtopors^{AA})$ (Secombe and Parkhurst 2004); and two heterozygotes $dtopors^{f05115}$/*SM1*, *Cy* and $Df(dtopors^{AA})$/*SM1*, *Cy*. All were collected < 24 hrs after eclosion and kept at room temperature (RT). At day one of age, all the testes sizes appeared to be of the same size (Figure 2). At ten days of age, there were no noticeable qualitative differences between either of the heterozygote classes and wildtype (Figure 2). However, both the *dtopors* mutant testes appear to have a slight deviation in testes size. However by 20 days of age, the difference between testes of the *topors* mutant classes and the heterozygotes/wildtype testes becomes more evident (Figure 2). The *topors* testes were smaller, appear shriveled and look more like wildtype and heterozygote testes from males of 40 days of age (Figure 2).

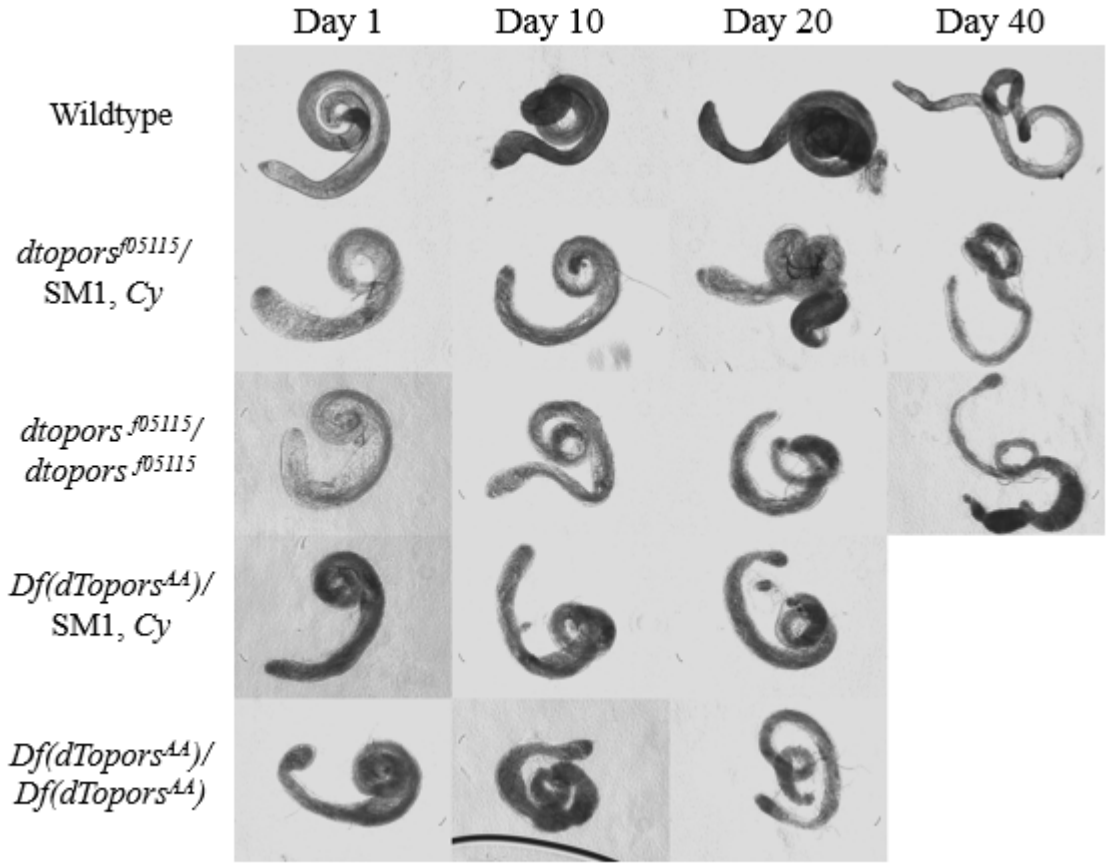

Figure 2. Phase contrast images of testes. The panel shows representative testes illustrating size changes with age for flies of the indicated genotype. Notable changes between wildtype and mutants occur at day 20.

Fecundity is reduced in dtopors males

Young male flies constantly attempt mating with virgin female flies and have the physical ability to inseminate multiple females in one day and copulation by males decreases with age (Miquel et al. 1976). Male fertility and number of viable offspring produced diminishes drastically by 12 weeks of age (Economos et al. 1979). Sperm

count does not seem to diminish until 40 days of age (Prowse and Partridge, 1997), so there is an apparent age-related change in physical capabilities or behavior as males age.

Since mutations in *dtopors* have been shown to lose volume in their testes at a premature age and produce fewer total offspring, I assayed male fertility to see if *dtopors* mutants' ability to produce offspring leveled off at a younger age than wildtype. Eight wildtype males, eight *dtopors*^{f05115}/*dtopors*^{f05115} males, and eight *dtopors*^{f05115}/*SM1*, *Cy* males were monitored over 30 days and their offspring counted. Within the first six days, fewer offspring from *dtopors* males vs. wildtype and *dtopors*^{f05115}/*SM1, Cy* heterozygotes were detected (Figure 3) (Table 1). I normalized the progeny produced between genotypes by dividing the original number of progeny produced from 0-6 days of age by the amount of progeny produced in subsequent six-day age ranges. Between the age of seven and twelve days old, *dtopors* males only produce 26% the amount of offspring they did from sexual maturity to six days old, whereas wildtype and heterozygote flies produced 77% and 93% of the original offspring, respectively (Figure 4) (Table 1). The trend continued from days 13 to 18 as *dtopors* males only produced 6% of their original number of offspring, whereas wildtype and heterozygotes flies produced 70% and 61% their original number of offspring, respectively (Figure 4) (Table 1). During the next age range from 19 to 24 days old, *dtopors* flies produced zero offspring, whereas wildtype and heterozygotes still produced 38% and 29% their original number of offspring, respectively (Figure 4) (Table 1). All male flies were monitored through 30 days of age, and wildtype and heterozygote flies were still capable of producing offspring (Figure 3 & 4) (Table 1).

dtopors flies exhibit high levels of nondisjunction as a result of chromatin condensation errors during meiosis I. This results in aneuploidy and consequentially fewer surviving progeny (Matsui, Sharma et al. 2011). To control for this defect, I normalized progeny production between genotypes by calculating progeny production as a percentage of the original progeny produced from day 0-6 in each genotype. My data support the conclusion that *dtopors* mutants produce significantly fewer offspring as they age. Also, the data suggest that *dtopors* mutants lose the ability to produce offspring at a much younger age because relative to wildtype flies, the percentage of original progeny produced by *dtopors* males on subsequent tested days dropped much faster.

Table 1. Average number of F1 progeny produced from crosses of wildtype females to *dtopors*^{f05115}/*dtopors*^{f05115} males; *dtopors*^{f05115}/*SM1*, *Cy* males; or wildtype males. Eight males of each genotype were monitored over 30 days and averages and standard deviations of numbers of progeny produced were calculated for each six-day interval.

	dtoporsf05115		*dtoporsf05115/Cy*		Wildtype	
Age of Male	Average # of F1	Standard Deviation	Average # of F1	Standard Deviation	Average # of F1	Standard Deviation
0 - 6	22.0	24.2	177.8	58.9	182.0	44.9
7 - 12	5.7	7.4	165.3	24.4	139.3	19.8
13 - 18	1.3	1.8	107.6	70.4	127.9	76.7
19 - 24	0.0	0.0	51.3	38.7	69.3	36.7
25 - 30	0.0	0.0	13.6	25.3	41.8	50.5

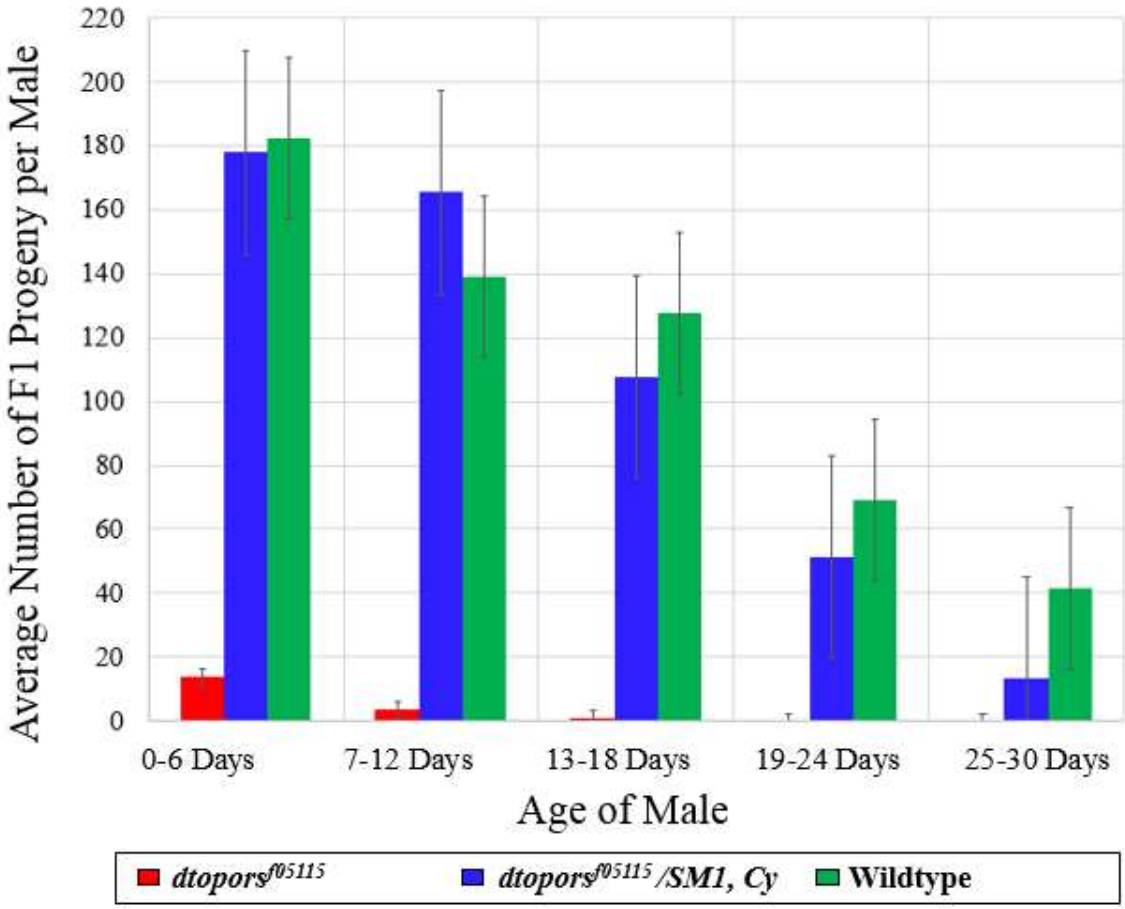

Figure 3. Average number of progeny +/- standard error produced by male flies of the indicated genotypes. Eight males of each genotype were monitored over 30 days and averages and standard deviations of progeny produced were calculated for each six-day interval.

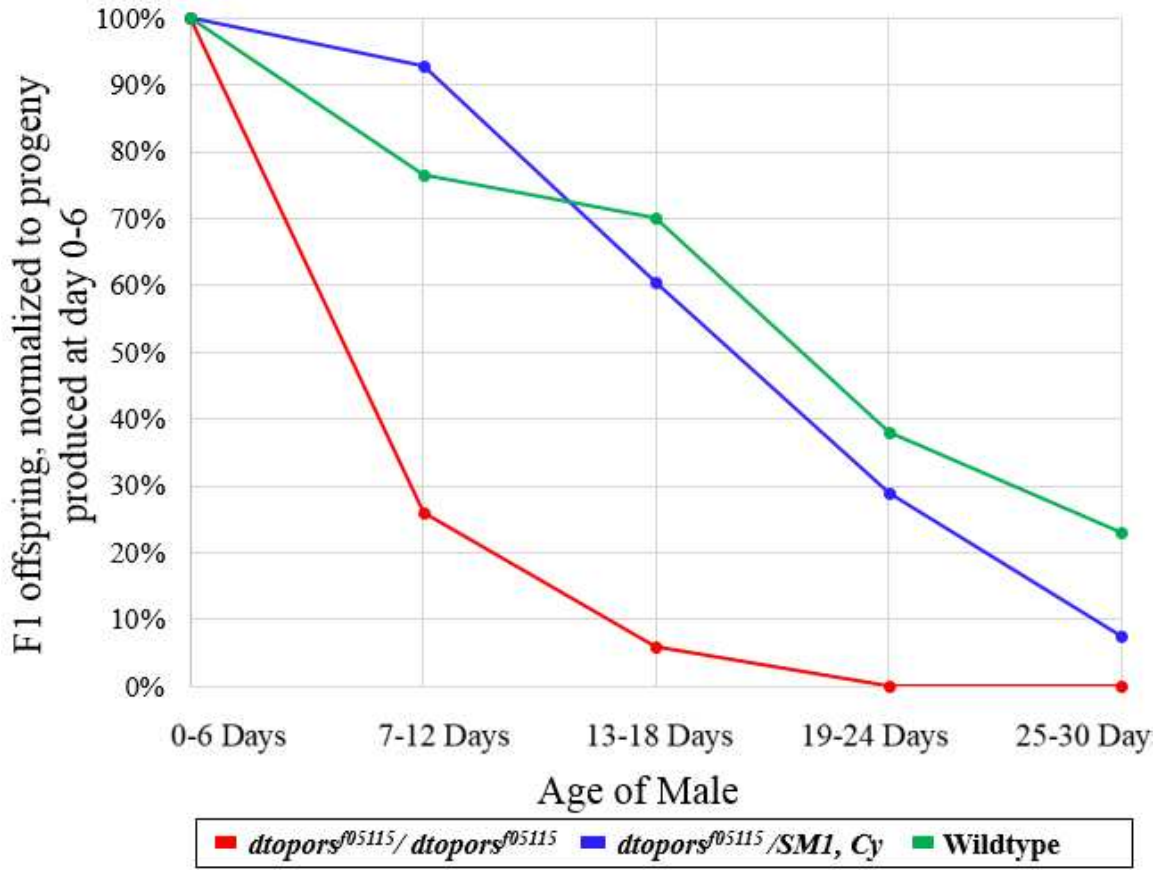

Figure 4. Age-related decline of fecundity of male flies through day 30 of age. Eight males of each genotype were monitored over 30 days, and the graph displays the average number of offspring produced for males of each age, normalized to progeny produced by 0-6 day-old males.

Germline stem cell number in aging dtopors males

It was possible that the age-related reduction in fecundity of *dtopors* males was due to an inability to maintain or regenerate germline stem cells. The fly testis is organized in a manner that facilitates direct examination of the germ line as stem cells, somatic cyst cells, and meiotic and post meiotic germline cells, which appear spatially ordered from the apical tip to the basal end of the testis. The stem cell niche surrounding

the hub typically contains 9-15 germ stem cells that come into physical contact with hub cells (Boyle, Wong et al. 2007). Previous studies show that there is a 25% decrease in germ stem cells in wildtype male flies by the age of 35 days (Wallenfang, Nayek et al. 2006), which was compared to *dtopors* stem cell counts.

The fly testis is organized in a manner that facilitates direct examination of germline stem cells, somatic cyst cells, and meiotic and post meiotic germline cells. The apical tip of each testis has a stem cell niche containing a set of 9-15 cells called the hub to which germ stem cells (GSC) adhere (Boyle, Wong et al. 2007). GSCs undergo an asymmetric division resulting in one cell remaining against the hub to keep stem cell identity, whereas the other cell becomes a gonialblast where it develops a spectrosome and begins differentiation. The gonialblast is surrounded by two cyst cells which also adhere to the hub and divide asymmetrically. Gonialblasts divide four times to form a cluster of 16 different interconnected spermatogonia that enter meiosis soon after the last mitotic division. Older spermatogonia and spermatocytes are shifted to the basal end of the testis as new stem cell divisions occur from the hub (Cheng, Tiyaboonchai et al. 2011).

To assay for premature germ cell loss in *dtopors*, I dissected wildtype, *dtopors*^{f05115} homozygotes, *dtopors*^{f05115}/*SM1*,*Cy* heterozygotes, and *dtopors*^{f05115}/+ heterozygotes that were less than one day old or ten days old. The ten day timepoint was chosen because *dtopors* males showed a significant reduction in progeny production by this age. Anti-fasciclin III was used to stain the membrane in the hub and Vasa antibodies were used to stain the cytoplasm of germline cells and provide visual

identification of cell type (Wong and Jones, 2012). Germline stem cells, which were identified as cells positive for Vasa staining that were in contact with hub cells, were counted in at least five different testes per genotype. All genotypes examined had similar numbers of germline stem cells at both days tested (Figure 5) (Table 2).

Data suggests there is no significant reduction in germ stem cells that occurs early in life in *dtopors* flies.

Table 2. Germ stem cell counts per testis on Day 1 and Day 10 of age. Averages and standard deviations were calculated from five individual testis per genotype at each age.

	Wildtype		***dtoporsf05115/SM1, Cy***		***dtoporsf05115/dtoporsf05115***	
Age	**Average # of Cells**	**Standard Deviation**	**Average # of Cells**	**Standard Deviation**	**Average # of Cells**	**Standard Deviation**
1 Day	**8.2**	**0.84**	**8.0**	**0.71**	**7.2**	**0.45**
10 Days	**7.4**	**0.55**	**7.4**	**0.55**	**6.8**	**0.84**

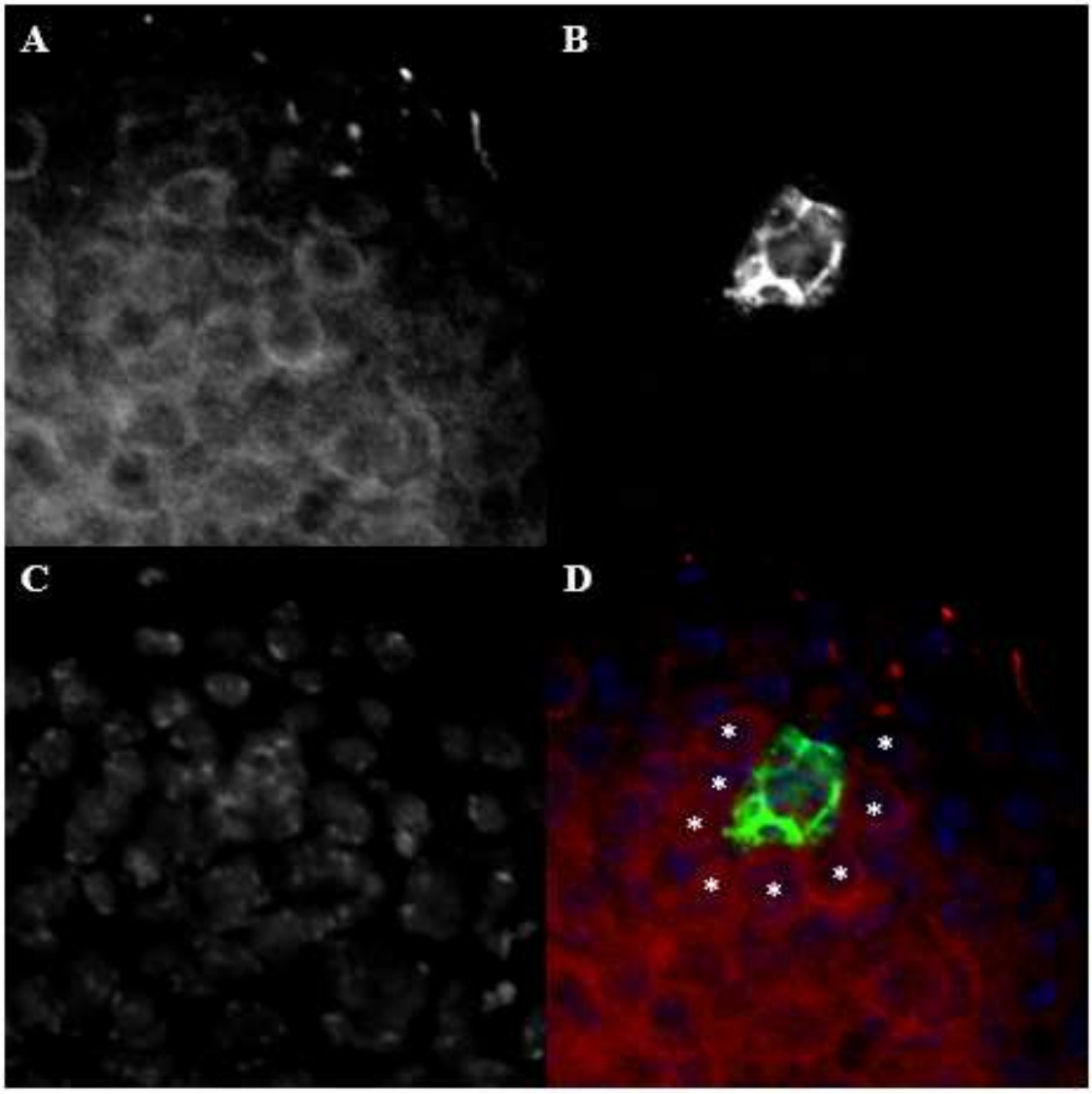

Figure 5. Confocal fluorescent micrograph of a one day old *dtopors*^{f05115} /*dtopors*^{f05115} testis. Panel A - germ cells stained with anti-Vasa; Panel B - hub cells stained with anti-Fasciclin III; Panel C – DNA stained with DAPI; Panel D overlay of anti-Vasa (Red), anti-Fasciclin III (Green), DAPI (Blue). Asterisks indicate germ stem cells in contact with the hub (D).

Lifespan is decreased in dtopors males

Longitudinal studies of aging in *Drosophila* have led to many discoveries regarding conserved genetic pathways and common environmental factors that influence lifespan (Kenyon, 2001). In assaying rapid age-related functional phenotypes associated with *dtopors*, it is pertinent to know if the overall life span of the flies themselves is shortened. *Drosophila melanogaster* wildtype flies have a maximum lifespan of 50-80 days (Grotewiel, Martin, et al. 2005), and I wanted to determine if the *dtopors* mutation would lower this lifespan maximum in a controlled environment.

To compare lifespans, I collected 500 males (ten per vial), < 24 hours after eclosion (emergence from pupa casing), of the following genotypes: wild type (Canton S) +/+; homozygotes $dtopors^{f05115}/dtopors^{f05115}$, and heterozygotes $dtopors^{f05115}/SM1,Cy$. To keep environmental stress to a minimum, the collection day was the only time that the flies underwent carbon dioxide anesthesia. Only males were used, and flies were not allowed to mate during their lifetime. Flies were transferred to fresh food every five days and the same food batches were used for all genotypes. The survivors were scored every five days. I realized early in the experiment that the $dtopors^{f05115}/SM1,Cy$ heterozygotes dominant wing defect was causing the flies to become stuck in the food and on the side of tube. Since these premature deaths were a result of another phenotypic defect other than *dtopors*, I discarded $dtopors^{f05115}/SM1,Cy$ class data from the experiment.

I followed the survivorship through 40 days of age since that age falls before the maximum life span age, and therefore many wildtype flies should still be alive at this point. After the first five days of life, there was no significant difference between

wildtype and *dtopors* (Figure 6) (Table 3, $P < 0.05$). However from day six on, there was a significant difference between wildtype and *dtopors* at every five day period (Figure 6) (Table 3, $P < 0.05$). The *dtopors* male flies perish at a faster rate with over 40% of the flies dead by 40 days of age, whereas the control flies still had over 80% of flies alive at day 40 (Figure 3).

The data shows that the rapid aging phenotypes associated with *dtopors* negatively affect the overall longevity. The significant difference between the viability of wildtype and *dtopors* flies in the same controlled environment begins as early as six days of age and continues through the test age of 40 days.

Table 3. Lifespan of *dtopors* males. 500 male flies (ten per vial) per genotype were scored for survival every five days. At ten days of age, there is a statistically significant difference between the number of wildtype and $dtopors^{f05115}/dtopors^{f05115}$ survivors ($P < 0.05$).

	Wildtype	$dtopors^{f05115}$ / $dtopors^{f05115}$
Day 0	500	500
Day 5	496	491
Day 10	488	467
Day 15	484	447
Day 20	476	414
Day 25	473	382
Day 30	463	339
Day 35	437	316
Day 40	412	292

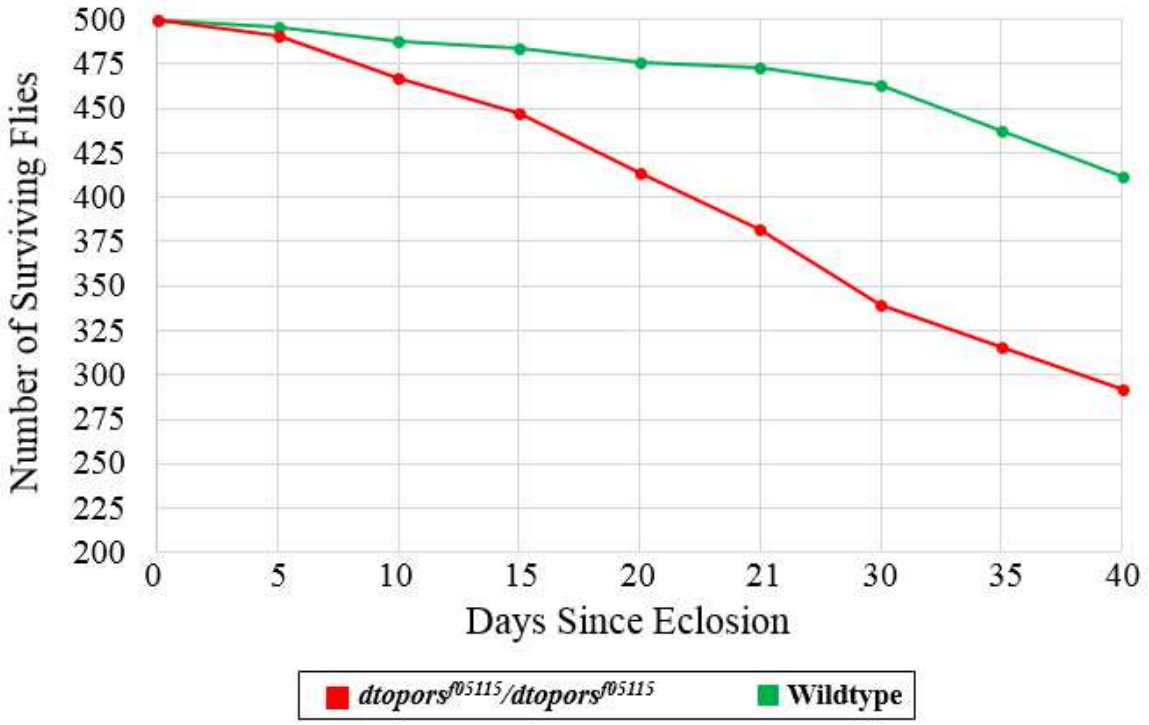

Figure 6. Survivorship curve of *dtopors*^{f05115}/ *dtopors*^{f05115} and wildtype flies. 500 male flies per genotype were scored for survival every five days. Survival of the two genotypes significantly differed at ten days of age and on every subsequent day tested.

dtopors males show an age-related decline in negative geotaxis

Negative geotaxis is a locomotor behavior and innate escape response that causes flies to climb against gravity to the top of their container. The flies complete this response by running up the side of the container, which provides an opportunity to assay the correlation between behavior and/or physical ability and aging. Miquel et al. (1976) first reported that the percentage of flies that can reach a pre-determined height on a container wall declines with age. This decline in negative geotaxis also is correlated to varying temperatures, which indicates the senescence of fly negative geotaxis is related to physiological age and not chronological age (Helfand and Rogina, 2000).

To see if a mutation in *dtopors* has any effect on flies' ability to perform negative geotaxis, I used a modified version of Miquel et al. (1976) locomotory assay. Male flies of the following genotypes: wildtype (Canton S) +/+; $dtopors^{f05115}/dtopors^{f05115}$; $Df(dtopors^{AA})/Df(dtopors^{AA})$, $dtopors^{f05115}/Df(dtopors^{AA})$ and a $dtopors^{f05115}/+$ were collected < 24 hrs after eclosion and kept at room temperature (RT). I also tried assaying the $dtopors^{f05115}/SM1, Cy$ heterozygote, but unfortunately the dominant phenotypic marker *Cy* on the balancer chromosome in the heterozygotes appeared to interfere with the assay. I noticed that the curly wing phenotype was having a negative effect on flies' ability to complete the climb to the top of the tube wall, even in very young flies. Therefore, I eliminated this dataset from our analysis.

Wildtype flies and $dtopors^{f05115}/+$ both completed the task of reaching the mark in less than two min both at day one and day ten after eclosion. $dtopors^{f05115}/dtopors^{f05115}$ and $dtopors^{f05115}/Df(dtopors^{AA})$ both showed a significant reduction in negative geotaxis on day one as only 80% and 82%, respectively, complete the task (Figure 7) (Table 4, $P < 0.05$). $Df(dtopors^{AA})/Df(dtopors^{AA})$ displayed a reduced percentage of flies completing the task at day one as only 88% complete but is not a significant decrease. At day ten, the trend is even worse for $dtopors^{f05115}/Df(dtopors^{AA})$ and $dtopors^{f05115}/dtopors^{f05115}$ as only 56% and 52% of flies complete the task, whereas $Df(dtopors^{AA})/Df(dtopors^{AA})$ do not show a significant decrease in percentage of flies that complete the task from day zero to day ten (Figure 7) (Table 4, $P < 0.05$). Wildtype flies and $dtopors^{f05115}/+$ both showed a significant age-related decrease after day 20 of age as only 86% and 78% completed the task (Figure 7) (Table 4, $P < 0.05$). Only 30% of $dtopors^{f05115}/$

dtopors^{f05115}, 42% of *dtopors*^{f05115}/*Df(dtopors*^{AA}*)*, and 48% of *Df(dtopors*^{AA}*)*/ *Df(dtopors*^{AA}*)* completed the task at 20 days of age, a significant reduction compared to wildtype and *dtopors*^{f05115}/+ at 20 days of age (Figure 7) (Table 4, $P < 0.05$). Even though wildtype flies exhibit a decrease in negative geotaxis at day 20 of age, *dtopors* mutants exhibit a decline in negative geotaxis from eclosion and it seems to diminish at an abnormally faster rate over time.

In summary, a decrease in negative geotaxis occurred at an earlier age in *dtopors* flies than in wildtype. This data suggests that there is either an accelerated senescence of either musculature or neural response in *dtopors*.

Table 4. Decline of negative geotaxis in *dtopors* males. 50 flies per genotype were tested at one, 10, and 20 days of age.

Wildtype (+/+)			*Df(dTopors^{AA})/Df(dTopors^{AA})*			
Day 1	**Day 10**	**Day 20**	**Day 1**	**Day 10**	**Day 20**	
0	0	7	6	8	26	# Flies Did Not Complete
12.96	8.18	14.95	17.86	14.55	15.83	Average Completion (Sec)
10.14	3.45	17.92	16.91	13.26	14.82	Standard Deviation
100.0%	100.0%	86.0%	88.0%	84.0%	48.0%	% Flies Complete
dtopors^{f05115}/+			*dtopors^{f05115}/Df(dTopors^{AA})*			
Day 1	**Day 10**	**Day 20**	**Day 1**	**Day 10**	**Day 20**	
0	0	11	9	22	29	# Flies Did Not Complete
14.64	7.20	23.67	23.24	23.96	36.29	Average Completion (Sec)
16.09	7.22	19.49	17.21	14.65	22.12	Standard Deviation
100.0%	100.0%	78.0%	82.0%	56.0%	42.0%	% Flies Complete
			dtopors^{f05115}/dtopors^{f05115}			
			Day 1	**Day 10**	**Day 20**	
			10	24	35	# Flies Did Not Complete
			19.24	16.62	21.67	Average Completion (Sec)
			10.04	18.88	15.63	Standard Deviation
			80.0%	52.0%	30.0%	% Flies Complete

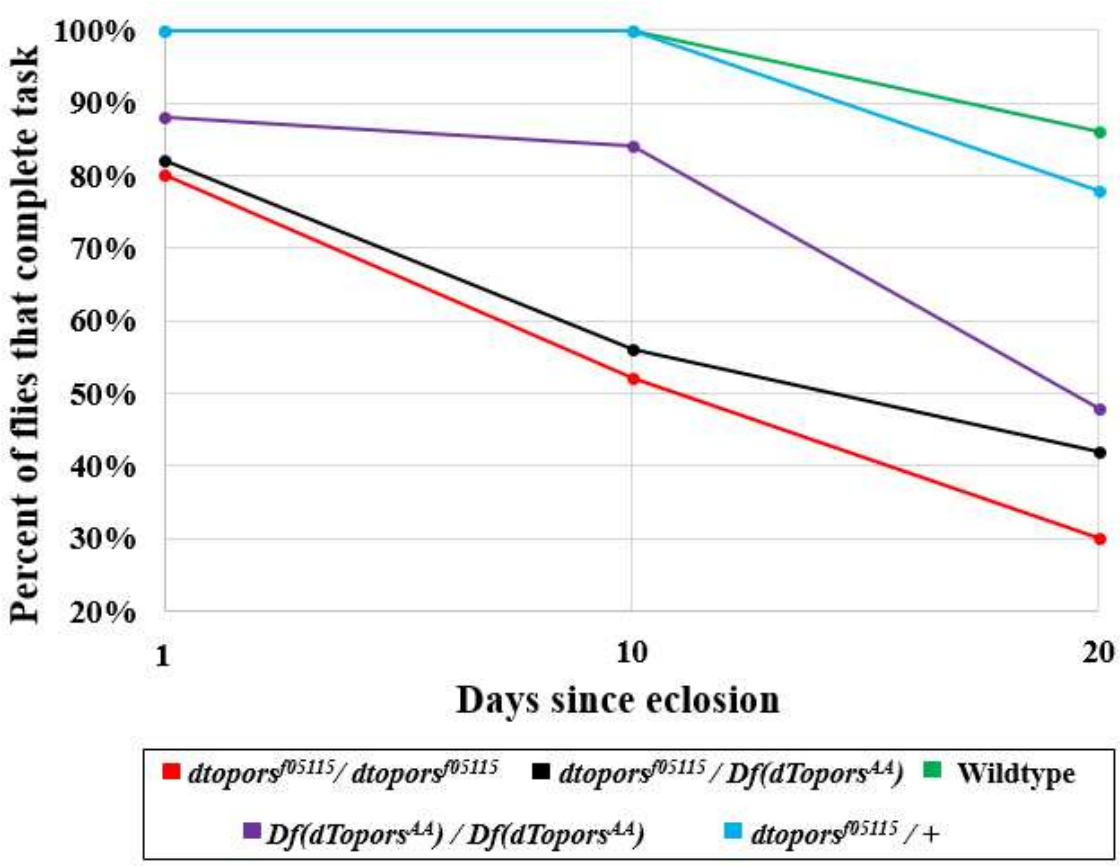

Figure 7. Average number of flies that complete the negative geotaxis assay. Fifty male flies of each genotype and age were tested and averages were calculated based on the flies' ability to complete the task.

Innate immunity is decreased in dtopors males

Drosophila have innate immune systems that consist of multiple defense mechanisms including the production of antibacterial proteins (DeVeale et al. 2004). Gene expression of these immune system proteins increases as flies age, inferring that the actual function of the immune system decreases over time (Grotewiel, et al. 2005). One specific *Drosophila* antibacterial protein diptericin peaks at six hrs after inoculation with live bacteria and is maintained at high levels for at least 48 hrs in one and two week old

flies. This response time is longer in three and four week old flies (Zerofsky, et al. 2005). Yet, total diptericin expression after inoculation is maintained at high levels in one to four week old flies with the highest expression in the four week old flies. The initial response time to bacterial infection in flies decreases with age causing flies to be unable to clear the live bacteria, which promotes persistent infections and antibacterial gene expression (Kim et al. 2001; Zerofsky, et al. 2005). *Drosophila* innate immune response occurs by both a Toll-like receptors (TLRs) dependent pathway, which controls host defense of gram-positive bacterial infection and a TLR independent pathway which controls host defense of gram negative bacterial infection. I tested both these pathways for *dtopors* related premature aging defects.

To determine if innate immunity is compromised at a younger age in *dtopors* mutants, I assayed the ability of mutants to survive infections with live bacteria. At least 30 flies of the following genotypes: wild type (+/+); $dtopors^{f05115}/dtopors^{f05115}$; and $dtopors^{f05115}/SM1, Cy$ at day 1, 15, and 30 since eclosion were injected with either the gram-positive *Staphylococcus aureus* (*S. aureus*) or the gram-negative *Escherichia coli* (*E. coli*). A mock injection was performed on at least ten flies of each genotype and age group and no significant loss of life occurred from the injection alone (data not shown). At day one of age, there was a significantly lower average number of survivors of *E. coli* injected $dtopors^{f05115}/dtopors^{f05115}$ compared to wildtype and $dtopors^{f05115}/SM1, Cy$ (Table 5A, $P < 0.05$) at all three time points. At day one of age, there was a significantly lower average number of survivors of *S. aureus* injected $dtopors^{f05115}/dtopors^{f05115}$ compared to wildtype but no difference between $dtopors^{f05115}/dtopors^{f05115}$ and $dtopors^{f05115}/SM1, Cy$

(Table 5B, $P < 0.05$) at all three time points. At 15 days of age, there is only a significant difference between the average number of wildtype and *dtopors*^{f05115}/*dtopors*^{f05115} survivors after 48 hours of injection with *E. coli.* At 15 days of age, there is no significant difference between the average number of wildtype and *dtopors*^{f05115}/*dtopors*^{f05115} survivors after injection with *S. aureus* (Table 5B, $P < 0.05$). There was a significant difference between the average survivors of wildtype and *dtopors*^{f05115}/*dtopors*^{f05115} mutants injected with *E.* coli and *S. aureus* at 30 days of age only at 72 hours after injection (Table 5A & 5B, $P < 0.05$). This trend is the same for *dtopors*^{f05115}/*dtopors*^{f05115} and *dtopors*^{f05115}/*SM1*, *Cy* (Table 5A & 5B, $P < 0.05$). The data does not support the expected age-related decline in flies' ability to survive infection with either bacteria strain in wildtype flies (Figure 8).

Prior research with *Drosophila* and innate immunity to bacteria provides data that as flies age, their immune system weakens (Grotewiel, et al. 2005), but this age-related decline was not evident in my data. I did find that *dtopors*^{f05115}/*dtopors*^{f05115} flies have a lower initial immunity.

Table 5. Average survival of flies injected with bacteria. Data shows percent of flies alive at 24, 48, and 72 hours post-injection with (A) gram negative *E. coli* or (B) gram positive *S. aureus.*

A Injected with *E. coli*			
1 Day Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	69.1%	61.8%	56.4%
dtopors^{f05115} / SM1, *Cy*	70.9%	67.3%	63.6%
dtopors^{f05115} /*dtopors*^{f05115}	38.0%	36.0%	30.0%
15 Days Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	53.8%	28.8%	28.8%
dtopors^{f05115} / SM1, *Cy*	79.6%	70.4%	38.9%
dtopors^{f05115} /*dtopors*^{f05115}	60.0%	50.9%	27.3%
30 Days Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	80.0%	57.1%	51.4%
dtopors^{f05115} / SM1, *Cy*	85.7%	78.6%	69.6%
dtopors^{f05115} /*dtopors*^{f05115}	64.5%	51.6%	22.6%

B Injected with *S. aureus*			
1 Day Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	76.4%	36.4%	23.6%
dtopors*^{f05115}/SM1, *Cy	70.6%	33.3%	15.7%
***dtopors*^{f05115}/*dtopors*^{f05115}**	61.5%	25.0%	9.6%
15 Days Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	51.0%	2.0%	2.0%
dtopors*^{f05115}/SM1, *Cy	69.6%	1.8%	1.8%
***dtopors*^{f05115}/*dtopors*^{f05115}**	39.2%	2.0%	2.0%
30 Days Old	**24 Hours**	**48 Hours**	**72 Hours**
Wildtype	83.9%	25.8%	25.8%
dtopors*^{f05115}/SM1, *Cy	75.8%	36.4%	27.3%
***dtopors*^{f05115}/*dtopors*^{f05115}**	66.7%	28.9%	6.7%

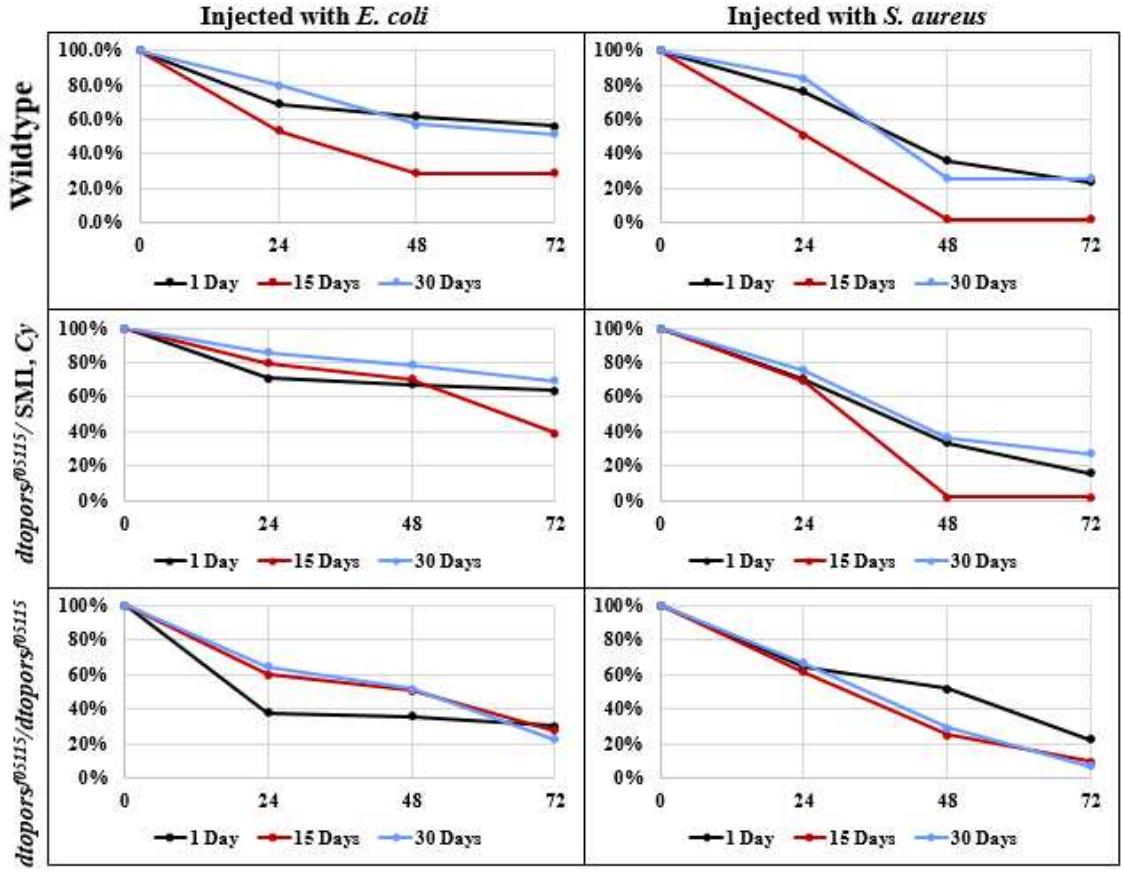

Figure 8. Average survival of bacteria-injected flies. Wildtype +/+, *dtopors*^{f05115} /*dtopors*^{f05115} and *dtopors*^{f05115}/*SM1, Cy* were injected and monitored after 24, 48, and 72 hours for mortality.

Discussion

Analysis of our results indicate that mutations in *dtopors* can affect aging in both the germ line and soma of male flies, however, not all tissues are equally affected. Germ stem cell number does not appear to be reduced at a younger age, yet *dtopors* testes appear smaller and less full. Longevity and fecundity are shorted in *dtopors* males as there is a significant difference compared to wildtype. Negative geotaxis also declines faster. Innate immunity is initially lower in *dtopors* males, but no age-related decline is

apparent. Rapid aging appears to be cell specific as no direct age-related correlation was found between *dtopors* and innate immunity to bacterial infection. However, our data contradicts prior findings that the *Drosophila* immune system becomes less able to defend bacterial infection over time (Kim et al. 2001, Zerofsky et al. 2005). Our data also shows that a functional copy of *dTopors* is required for standard aging of fecundity, mobility, and longevity in male *Drosophila*. The decline in fecundity may be the result of slower division of germ stem cells in the testis, as no differences in the overall number of stem cells were observed. The difference in mobility could be attributed either to neural or muscular senescence.

dtopors and the germ line

The fly testis has evolved in an organized manner where functional spermatocytes are a product of both mitosis and meiosis and originate from germ stem cells (Boyle, Wong et al. 2007). A germ stem cell receives internal cues that causes it to divide and one cell remains in contact with the control hub while the other cell begins the differentiation cascade to a sperm (Cheng, Tiyaboonchai et al. 2011). Nonfunctional dTopors has previously been linked to errors in meiotic chromosome segregation, nuclear lamina defects, and reduced fertility (Matsui, Sharma et al. 2011), and prior information (M. Matsui, J. Tomkiel, personal communication) suggested that the testes of *dtopors* males may lose volume earlier in life than wildtype flies.

Upon examining *dtopors* testes, there appeared to be no qualitative difference compared to wildtype in the size of the testes of one day old flies. After ten days, there

was a noticeable difference in the appearance of the testes of two different *dtopors* mutants and wildtype, which were even more prevalent in 20 day old flies whose testes appeared more like wildtype testes of 40 day old males. Male flies begin to lose sperm count around age 40 (Prowse and Partridge, 1997) partially due to the decline in stem cell renewal within the niche which reaches a 35% loss of in wildtype males by 50 days of age (Wallenfang, Nayek et al. 2005). Because I saw no difference in numbers of germline stem cells in aged *dtopors* flies, it is unlikely that the diminished testis size results from a failure to replenish stem cells.

There are multiple possibilities to explain why *dtopors* males might retain wildtype stem cell number, yet have diminished testes size and faster fecundity. First, some of the difference in testes volume could be a result of higher levels of apoptosis due to the nuclear abnormalities. This possibility could be tested by performing TUNEL assay on *dtopors* and wildtype flies and compare the number of germline cells undergoing death. If higher apoptosis is a correct hypothesis, it would answer why the testes total area would appear to be lower than wildtype at each age but does not answer why the area of the testes seems to decrease more rapidly/age faster at each interval examined. A second possibility is the difference could result from slower stem cell differentiation. The nuclear lamina defects may interfere with the differentiation signals sent to the stem cell, cyst cell or both. This possibility could be tested by knocking out a component of the signaling pathway and see if the effects are similar to when *dtopors* is mutated. A final possibility is that the germ or cyst stem cells themselves undergo the same types of chromatin/nuclear lamina defects seen later in differentiation during

prophase I. Unresolved chromatin interactions could slow the ability of the daughter stem and/or cyst cell to separate. LEM (LAP2, emerin, MAN1) domain (LEM-D) proteins are components of the nuclear lamina, and prior work with a specific LEM-D Otefin shows that mutations in the coding gene causes loss of germ stem cell in female *Drosophila* (Barton, Pinto et al. 2013). Since dTopors also interacts with the nuclear lamina, perhaps stem cell loss could still be a possibility and perhaps not evident in my sample size.

dtopors male flies have been shown to produce fewer progeny than wildtype males (Matsui, Sharma et al. 2011), and I wanted to determine if the declining testes sizes seen would be associated with faster senescence of fecundity. Wildtype males reproductive capability become minimal by 12 weeks of age (Economos, Miquel et al. 1979), and this age-related reduction is not because of sperm production loss (Prowse and Partridge, 1997) but more likely a result of physical limitations and/or behavioral changes. We observed a diminished number of progeny from *dtopors* males beginning at sexual maturity, however there was also a substantial loss of fecundity much earlier in life. *dtopors* males were completely sterile by 19 days of age, possibly from stem cell differentiation errors, interference in signaling pathways, neither or both. There is also the possibility that behavioral responses aged rapidly causing flies to lose the physical urge to mate, however male *dtopors* flies were seen copulating past the date of sterility.

As there are many parallel consequences of nuclear lamina disruption in *dtopors* mutants and HGPS/laminopathies in humans, study of the germline cells in *Drosophila* may provide insight into why HGPS patients never reach sexual maturity (Varga,

Eriksson, et al. 2006). It has been shown that progeria and age-related nuclear defects also occur in human somatic stem cells causing cell dysfunction (Scaffidi and Misteli, 2008), so the possibility that rapid aging also occurs in the germ cells is likely. The germline stem cells may be completely diminished by sexual maturity, which would explain failure to thrive. Another possibility is that in progeria the decrease in germ cell production at sexual maturity may reflect a conserved evolutionary regulatory mechanism. Mammals (Gu, Fan et al. 2011), flies and roundworms (Hsin and Kenyon, 1999) can all prolong life by depleting germ cell production under varying conditions of stress. This alteration of energy expenditure allows longevity in order to survive until conditions better. *C. elegans* can reverse this effect and regenerate germ cells when conditions are stable (Hsin and Kenyon, 1999). The combined stress applied on the body from the nuclear lamina defects and the destruction it causes may warrant regulation of stem cell production, yet never regains germ stem cell growth because of the fatal effects the nuclear lamina defects and cell instability causes.

dtopors and the soma

I wanted to see if there were also indications of early aging continued in somatic cells of *dtopors* mutants. As an initial assessment, I asked if longevity was negatively affected by *dtopors*. I monitored 500 male flies of both wildtype and $dtopors^{f05115}$/ $dtopors^{f05115}$ and found a significant decrease in lifespan in the *dtopors* mutants as only 58% of *dtopors* males were alive after 40 days and 82% of wildtype flies remained alive. The average fly lifespan is 50 to 80 days (Groteweil, Martin et al. 2005) so the premature

death rate is significant. This longevity test does not specifically identify the somatic cell types affected by the nuclear lamina disruption. In laminopathies in humans, multiple autosomal cell types are affected including but not limited to adipose, dermal, and muscle tissue (Varga, Eriksson et al. 2006), so it is possible that multiple autosomal cells are affected in *dtopors* mutants as well.

To better characterize specific autosomal cell types affected by *dtopors*, I performed tests of negative geotaxis, which is an innate response in flies that declines with age (Miquel, Lundgren et al. 1976). I found that *dtopors* mutations cause premature loss in ability to complete the task of negative geotaxis in male flies containing one of three different mutations. Wildtype and heterozygote flies had a minimal loss of flies able to complete the climb at age 20, which is expected since the natural decline in negative geotaxis begins as early as 14 days of age (Miquel, Lundgren et al. 1976). *dtopors* had lower ability to complete the task from day one. This suggests that loss in geotaxis is not solely due to rapid aging since the rate is lower from eclosion. However, as *dtopors* males age, the rate of flies able to complete the task decreases much faster than wildtype. There were also notable cases of *dtopors* falling to the bottom of the vial during the trek to the top. There is slight variation between the three different *dtopors* mutations which is likely due to sample size.

Revealing that *dtopors* flies lose the ability to fulfill the innate response of negative geotaxis still leaves unanswered questions. It is not possible to tell from the results of this test are caused by rapid aging of muscular activity or behavioral senescence. Loss of lamin C in *Drosophila* was recently shown to cause pupal

metamorphic lethality due to tendon defects and muscle defects. This phenotype closely mimics the human laminopathy Emery-Dreifuss muscular dystrophy (EDMD) which occurs as a result of human A-type lamin mutations. In flies, the tendon defects could be rescued by wildtype *lamin C* expression in tendon cells expressing the cytoskeletal protein Shortstop (Uchino, Nonaka et al. 2012). This rescue did not occur in skeletal muscle cells suggesting varying roles for lamin C in *Drosophila* musculature. Since laminopathies have been observed to affect the musculature, it is quite possible that *dtopors* mutants suffer from rapid muscle breakdown which would explain why their ability to complete the climb diminishes quicker. Additional muscular tests, such as total distance traveled or flight duration, or more direct examination of muscle could be performed to test this possibility.

It is also pertinent to analyze the possibility that there is additional premature behavioral senescence which would also play a role in the lack of ability to complete the task. Since the flies did not lose total ability to complete the task by 20 days old, perhaps if it is a neural decay, it may not have as fast an effect on the flies. This possibility mirrors the human disease of Parkinson's, which is a neural disease that causes premature slowing of movement and trouble walking (Leroy, Boyer et al. 1998). As flies age they incur natural deficits in behaviors such as locomotion, olfaction, learning, and circadium rhythm. These senesce at different ages throughout a fly's lifespan, and some are also affected by varying temperatures and other environmental factors (Miquel, Lundgren et al. 1976). Using a pattern of assays that have different initial senescence age ranges and

adjusting environmental factors would provide more insight into the cause of the rapid decline of negative geotaxis in male *dtopors* mutants.

dtopors and innate immuntity

Drosophila have an innate immune response to pathogens including the production of antibacterial proteins, which attack bacteria and clear it from the fly's system. Since various transcriptional machinery and peptides are needed for this defense, I was curious to see if *dtopors* males were less able to survive bacterial infection and if so, if this defense senesces faster over time. One of these fly antibacterial proteins diptericin has been identified to be overly expressed at around fly age of 28 days (Zerofsky, Silverman et al. 2005) so innate immunity seems to decline at this time.

I monitored flies after being injected with either *E. coli* or *S. aureus* for their ability to survive after 24, 48, and 72 hours. The younger *dtopors* flies appear to be less immune to the bacteria than the wild type and heterozygous flies, however there appeared to be no age related rapid decline in *dtopors* flies' ability to survive the bacterial infection. Also the wildtype flies injected with *E. coli* at 30 days of age survive better than flies of 15 days of age. Since this result differs from that of previous work (Zerofsky, Silverman et al. 2005), I think the sample size of injected classes may have been too small or unequal injection volumes of bacteria have skewed this assay. No evidence was apparent of age-related decrease in innate immunity, but this assay should be repeated with more proficient injection technique.

dtopors: A Drosophila model for Progeroid disease?

Longevity, behavioral and motor function, and fecundity all appear to rapidly age when mutations in *dtopors* are present. dTopors is clearly necessary for wildtype nuclear lamina formation and stability, which may be the culprit of the aforementioned rapid aging phenotypic anomalies. Preliminary results show that innate immunity is not affected but should be further investigated.

A correlation between rapid aging in *dtopors* male flies and laminopathies in humans is possible. Rapid aging phenotypes in humans with HGPS are directly related to disruption in the lamina composition. Despite the fact that hTopors has not been shown to localize to the nuclear lamina, there are multiple functions for hTopors that are mechanistically similar to dTopors. In humans, Topors was shown to function as a SUMO-1 E3 ligase for mSin3A and other chromatin modifying proteins (Pungaliya, Kulkarni, et al. 2007). Perhaps it is the effects Topors has on chromatin proteins that is the key to the weakening of nuclear architecture and misregulation of aging genes in humans. Even though sumoylation activities for dTopors are not evident, perhaps dTopors interacts with chromatin in a different manner in *dtopors* spermatocytes as well, and the laminar localization of dTopors may play no role in the blebbing defects. Laminopathies are still not well understood and preliminary results stated here are encouraging that *dtopors* mutant males may in at least some ways act as a model to study nuclear lamina defects and corresponding rapid aging phenotypes.

CHAPTER III

CONSTRUCTION OF A *DTOPORS/HTOPORS* EXPRESSION VECTOR

Materials and Methods

Competent cells

The Scott-Simanis transformation protocol (M. Montiero, personal communication) was used to prepare competent *E. coli* cells. A frozen stock of DH5α *E. coli* cells was streaked on a Ψ_a plate (Bacto-yeast extract, Bactotryptone, $MgSO_4*7H_2O$, pH 7.6, BactoAgar) and the plate incubated at 37° overnight. The following day a single colony was picked and used to inoculate 5 ml Ψ_β (Bacto-yeast extract; Bactotryptone, $MgSO_4*7H_2O$, pH 7.6) media and incubated overnight at 37°C at 250 rpm. On the third day, the culture was transferred into 500ml of Ψ_β media and incubated at 37°C and 250 rpm for approximately two hrs until the OD_{590} reached 0.48. Cells were chilled on ice for 5 min and then centrifuged at 4°C and 6000 rpm for 5 min. Cells were carefully resuspended in 100ml of ice cold TfbI (30mM potassium acetate, 100mM $RbCl_2$, 10mM $CaCl_2$ * $2H_20$, 50mM $MnCl_2$ * $4H_20$, 15% glycerol (v/v), pH 5.8) with pre-chilled pipettes and placed on ice for 5 min. The cells were again centrifuged at 6000 rpm for 5 min at 4°C. The pellet was resuspended in 12.5 ml of ice cold TfbII (10mM MOPS, 75mM CaCl2*$2H_20$, 10mM $RbCl_2$, pH 6.5, 15% glycerol (v/v)) and placed on ice for 15

min. 200 µl aliquots of the competent cells were stored in 2 ml Eppendorf tubes. The aliquots were placed in liquid nitrogen before being stored at -80°C.

Bacterial transformation

Tubes of 100 µl each of competent DH5α cells were thawed by hand and immediately placed on ice. The expression plasmid DNA was placed in a tube of cells and incubated on ice for 20 min. Cells were then heat shocked at 42°C for 2 min and returned to ice. 100 µl of sterile Luria broth (LB; Bacto-tryptone, Bacto-yeast extract, 10g NaCl, pH 7.0) was added to the tube and incubated for 1hr at 37°C. Cells were then plated on LB with Ampicillin plates (LB +AMP; Bacto-tryptone, Bacto-yeast extract, NaCl, Bacto-agar, Ampicillin (final concentration of 100 µg/ml), pH 7.0) and incubated overnight at 37°C.

Vector preparation

The bacterial artificial chromosome (BAC) attB-p[acman]-ApR-1245 (Venken, He et al. 2006), henceforth referred to as *p[acman]*, is an eukaryotic expression vector that expresses the ampicillin resistance gene and contains a phiC31 phage attB site that can be efficiently integrated by site-directed recombination into the fly genome at a specific attP site. *p[acman]* was obtained from the Drosophila Genome Resource Center (DGRC). The frozen sample was used to inoculate 500 ml LB+AMP (100 mg/ml) + BAC Autoinduction Copy Control Solution (CCS; Epicenter, Madison, WI) and incubated at 37°C overnight at 250 rpm. Cells were pelleted and *p[acman]* plasmid DNA

was purified via QIAGEN plasmid maxi kit #12163 (Qiagen, Alameda, CA). Plasmid DNA quantity estimated and DNA quality verified on a 0.7% agarose gel. DNA was quantified by a Nanodrop Spectrophotometer (Thermo Scientific) at an absorbance of 260 nm.

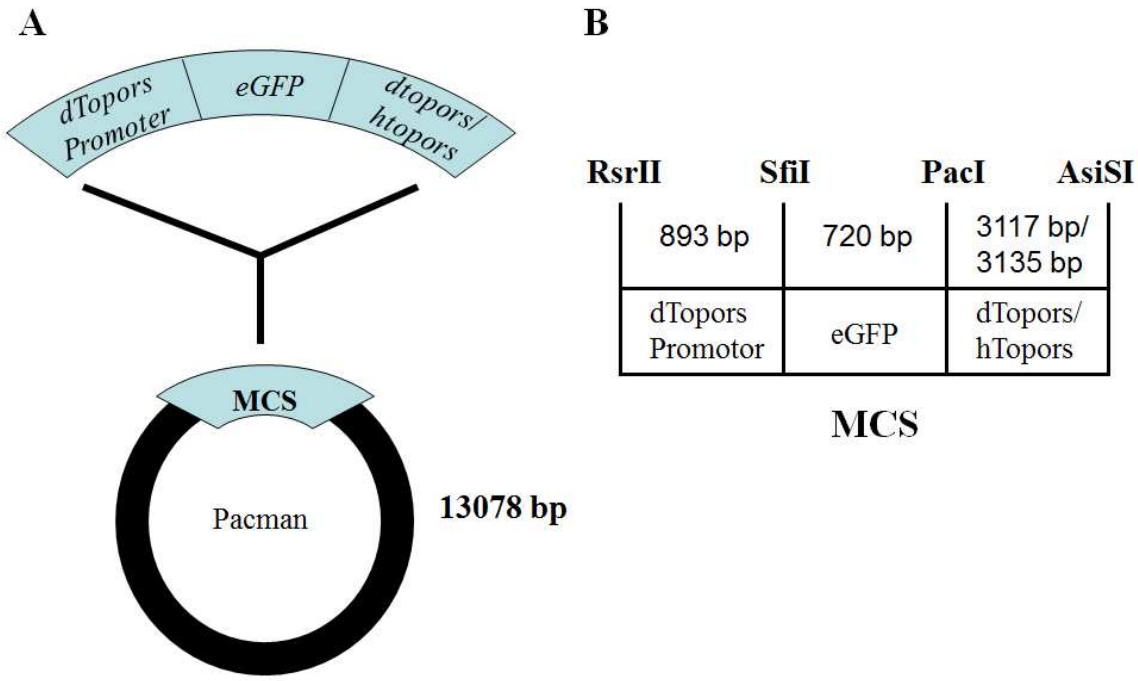

Figure 9. *attB-p[acman]-ApR-1245* diagram. The subcloning scheme (A) and digest specifics (B).

PCR amplification

DNA sequences corresponding to the *dTopors* promoter DNA, as predicted by Berkeley Drosophila Genome Project (University of California, Berkeley, CA) was amplified by PCR from a stock isogenic for chromosome 2. The Enhanced Green Fluorescent Protein (*eGFP*) cDNA was PCR amplified from pCasperhsGFPdTopors1-

1038FL (Byungura, 2009), a modification of a commercially available- pCasper plasmid (Clontech, Mountain View, CA). Specific oligonucleotide primers for both upstream and downstream sequences were designed for each gene and used for amplification (Table 5). PCR amplification cycling conditions were set at 30 cycles of denaturation at 95°C for 30 sec, annealing at varying temperatures (Table 5) for 30 sec and elongation at 72°C for 30 sec. All primers were purchased from Eurofins MWG Operon (Huntsville, AL). PCR products were purified with a QIAquick PCR purification kit # 28106 (Qiagen, Alameda, CA) and DNA quantity verified by agarose gel electrophoresis before and after purification.

Gene cloning

eGFP cloning into the p[acman] vector

p[acman] purified plasmid DNA was digested with *Sfi*I and *Pac*I restriction enzymes (Promega) respectively in two separate reactions under standard conditions. After each digest, *p[acman]* was purified on an agarose gel to remove the uncut molecules from the digest and purified with a QIAquick gel extraction kit #28704 (Qiagen, Alameda, CA). *eGFP* DNA was digested with both enzymes as above but purified using a QIAquick PCR purification kit. Purified products were quantified using a Nanodrop spectrophotometer. Double digested *eGFP* was ligated into the double digested *p[acman]* at the multiple cloning site (Figure 9A & B) using 10x ligase buffer and T4 ligase (Promega, Madison, WI) overnight at 10° - 25°C. Ligation products were transformed into DH5α competent cells and incubated on LB+AMP plates overnight at

37°C. An individual colony was used to inoculate 4 ml LB+AMP media and incubated overnight at 37°C at 250 rpm. Plasmid DNA was extracted and purified according to the mini-prep protocol (Del Sal, Manfioletti et al. 1988) with cetyl ammonium bromide (CTab; 5 mg/ml) (Fisher, Hampton, NH) and lysozyme (5mg/ml) (Sigma, St. Louis, MO) and the DNA yield was quantified using a Nanodrop spectrophotometer. Successful ligation was assayed by a diagnostic restriction enzyme digest with *Kpn*I (Figure 9) and separation of products by agarose gel electrophoresis to test for the presence of the *eGFP* insert (Figure 10). The isolated mini-prep DNA was also used as a template in a PCR reaction with forward and reverse primers for *eGFP*. An *eGFP* positive clone was selected and used to inoculate 500 ml LB+AMP medium and incubated at 37°C overnight at 250 rpm. The plasmid DNA was isolated and purified using a QIAquick plasmid maxi kit. The DNA yield was quantified using a Nanodrop spectrophotometer and the plasmid DNA sent to Eurofins MWG Operon for DNA sequencing.

Table 6. Primer pairs and annealing temperatures used in PCR amplification of *dtopors* promoter and *eGFP*.

Target Sequence	Restriction Site	Primer Name	Primer Sequence (5' to 3')
***dTopors* Promoter**	*RsrII*	FdTopPro	AACGGTCCGGGATCCAAATTATGACCA
72°C	*SfiI*	RdTopPro	CTGGCCATTAAGGCCTCTGGCGACTATATAGCGGCT
eGFP	*SfiI*	eGFPF	CTGGCCTTAATGGCCAAGCTTATGGTGAGCAAGGGCGAG
72°C	*PacI*	eGFPR	CATTAATTAACTTGTACAGCTCGTCCAT

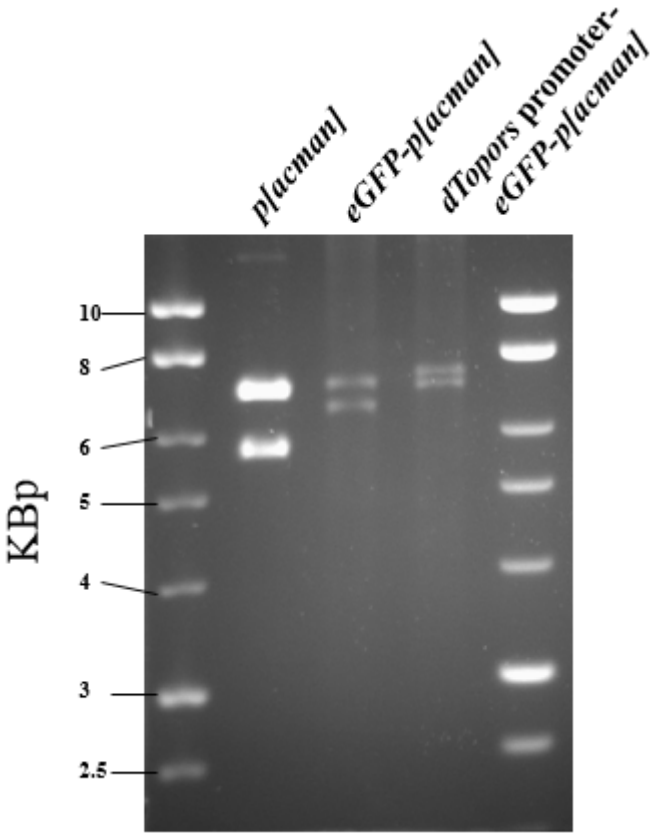

Figure 10. Diagnostic restriction enzyme digest of constructs with *KpnI*. Fragment pattern shows successful ligation of *eGFP* into *p[acman]* and *dTopors* promoter into *eGFP-p[acman]*.

dTopors promoter cloning into the eGFP-p[acman] vector

eGFP-p[acman] purified plasmid DNA was digested with *Rsr*II and *Sfi*I restriction enzymes (Promega) respectively in two separate reactions under standard conditions. After each digest, *p[acman]* was purified on an agarose gel to remove the uncut molecules from the digest and cleaned up with a QIAquick gel extraction kit. *dTopors* promoter cDNA was digested with both enzymes as above but purified using a QIAquick PCR purification kit. Purified products were quantified using a Nanodrop

spectrophotometer. Double digested *dTopors* promoter was ligated into the double digested *p[acman]* at the multiple cloning site (Figure 9A & B) using 10x ligase buffer and T4 ligase (Promega) as above. Ligation products were transformed into DH5α competent cells and incubated on LB+AMP plates overnight at 37°C. An individual colony was used to inoculate 4 ml LB +AMP media and incubated overnight at 37°C at 250 rpm. Plasmid DNA was extracted and purified according to the mini-prep protocol (Del Sal, Manfioletti et al. 1988), and the DNA yield was quantified using a Nanodrop spectrophotometer. Successful ligation was assayed by a diagnostic restriction enzyme digest with *Kpn*I and separation of products by agarose gel electrophoresis to test for the presence of the *dTopors* promoter insert (Figure 10). The isolated mini-prep DNA was also used as a template in a PCR reaction with forward and reverse primers for *dTopors* promoter (Figure 11). A *dTopors* promoter positive clone was selected and used to inoculate 500 ml LB+AMP medium and incubated at 37°C overnight at 250 rpm. The plasmid DNA was isolated and purified using a QIAquick plasmid maxi kit. The DNA yield was quantified using a Nanodrop spectrophotometer and the plasmid DNA sent to Eurofins MWG Operon for DNA sequencing.

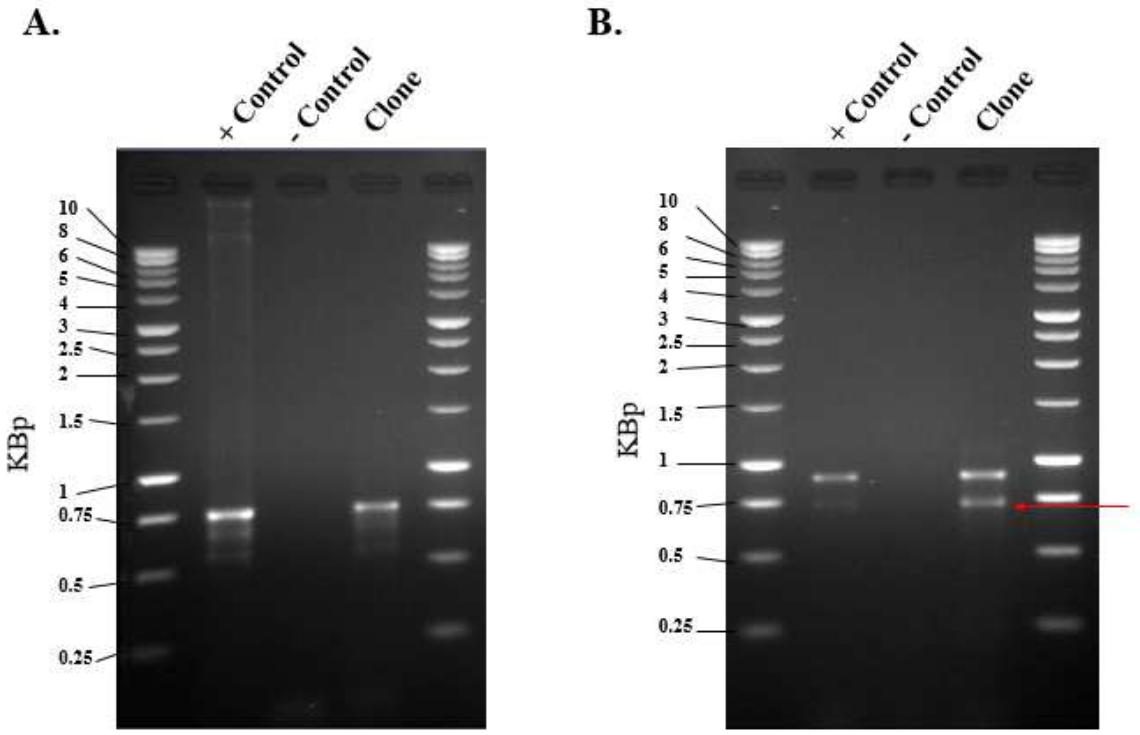

Figure 11. PCR reactions to test for ligation of insert into target location in *p[acman]*. (A) Ligation of *eGFP*. (+) a positive control template, pCasperhsGFPdTopors1-1038FL, (-) a negative control empty *p[acman]* vector and (clone) a positive clone. (B) Ligations of *dTopors* promoter. (+) Positive control is *iso cn br* genomic DNA, negative control (-) is *eGFP-p[acman]* vector and (clone) a positive clone. The lower band notated by the red arrow is a product of non-specific binding.

Results

I have created a transgene that contains the *eGFP* gene and the cDNA for the predicted region of the *dTopors* promoter. The *eGFP* gene lies downstream of the *dTopors* promoter to ensure that transcription eGFP mimics the pattern of endogenous *dTopors* expression. The *dTopors* promoter-*eGFP- p[acman]* vector is prepared so *dtopors* or *htopors* gene can be fused into the vector downstream of the promoter to produce a *topors-eGFP* transgene.

Discussion

Transgenes are useful tools because they can be used to introduce foreign DNA into organisms and compare differences with others that have the identical genetic background save the transgene. Using transgenes and vehicle controls, it can easily be determined if the introduction of a wildtype gene can rescue phenotypes seen in mutants, such as the aging effects seen here. Transgenes also allow expression in specific tissues and developmental stages to determine where or when temporally a protein is required for wildtype function. Lastly, transgenes help solidify that phenotypes have occurred as a result of a specific mutated gene and not in conjunction with another mutated gene.

A dtopors promoter-regulated expression construct

For future studies of *topors*, I made progress in creating a construct that will deliver a wildtype copy of *dtopors* to *dtopors* mutant flies. By expressing a wildtype copy of *dtopors* in a *dtopors* mutant background, it can be determined which specific age-related phenotypes can be rescued by a functional copy of *dtopors*. By keeping the genetic background identical, varying phenotypes between the *dtopors* mutant flies and transgenic *dTopors* flies can be stated with more confidence. Also the vector is prepared to have either a wildtype copy of *dtopors* or *htopors* ligated internally downstream of the *dTopors* promoter. The *eGFP* gene product can be used to study the localization of the wildtype copy of Topors in the mutant *topors* background.

C. elegans germ line cells have been shown to regulate the longevity of life by modulating pathways that regulate the ageing of the organism (Hsin and Kenyon, 1999).

Perhaps the somatic aging/germ cell defects seen in *dtopors* males work in a similar manner. The transgene will help answer some questions about specific tissue/tissue interactions since it can be used to express dTopors in different tissues at different times. By using regulated *Drosophila* promoters and other controlled gene expression methods like the GAL4-UAS system (Brand and Perrimon, 1993), the transgene can determine in what specific cells and at what developmental ages is Topors required to rescue longevity or any of the other rapid aging phenotypes.

As the phenotypes associated with *dtopors* mutations are better understood, *Drosophila* may serve as a model for better understanding connections between nuclear structure and aging.

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